Biogeographical diversity of plant associated microbes in arcto-alpine plants

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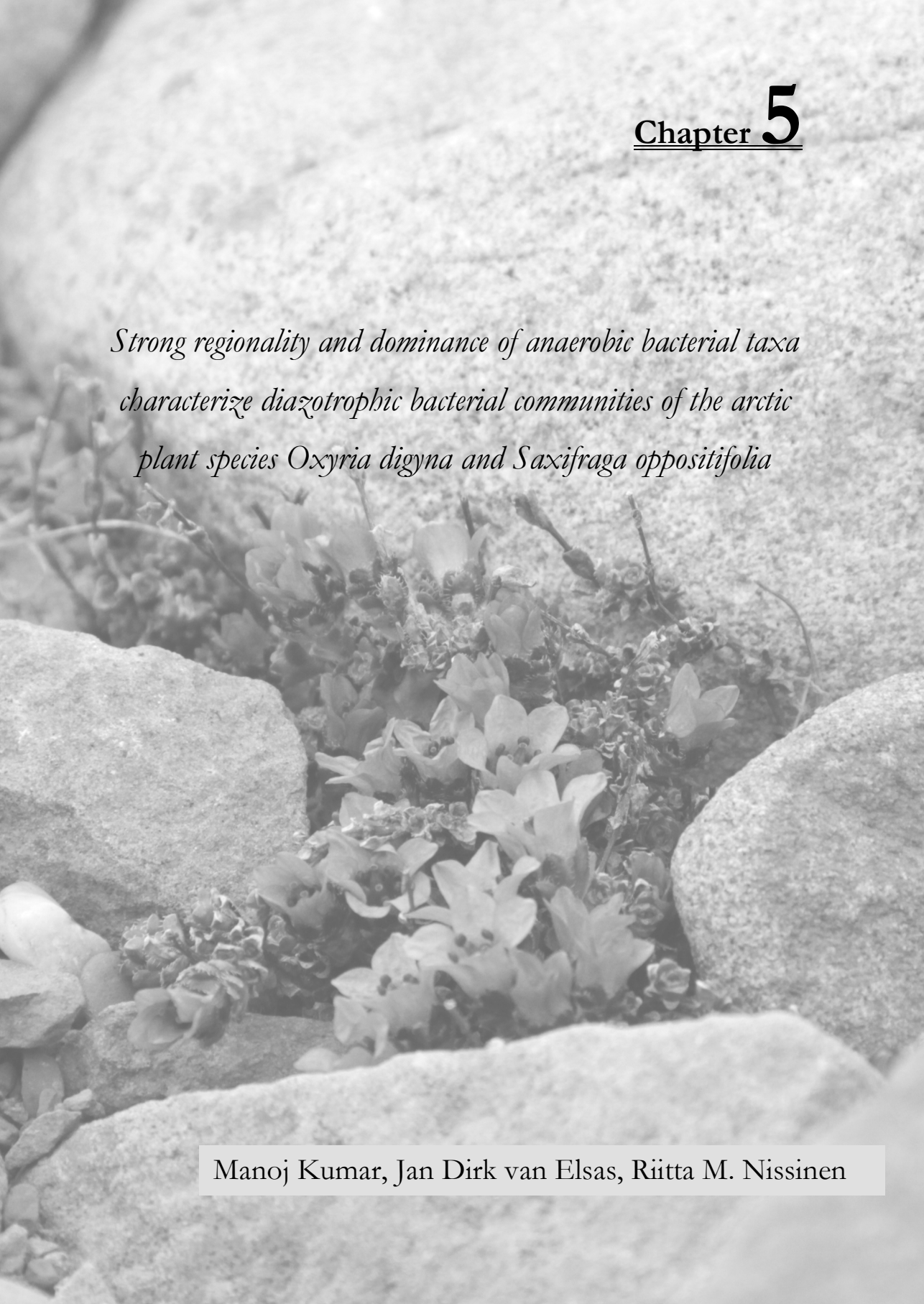

Chapter 5

Strong regionality and dominance of anaerobic bacterial taxa characterize diazotrophic bacterial communities of the arctic plant species Oxyria digyna and Saxifraga oppositifolia

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Abstract

Arctic and alpine biomes developing in rocky areas are most often strongly nitrogen-limited, and hence biological nitrogen fixation is a strong driver of such biomes. On the basis of total community DNA, we here studied the nitrogen-fixing communities at two arcto-alpine pioneer plant species, *Oxyria digyna* and *Saxifraga oppositifolia*, using the *nifH* gene as a proxy. Replicate samples taken from two arctic regions, Kilpisjärvi and Ny-Ålesund, next to one alpine region, Mayrhofen, were included, to study a South-to-North cold-climate soil gradient. The data revealed strong regional effects on the dominating nitrogen fixers. As a major overall finding, anaerobic bacterial taxa dominated the diazotrophic bacterial communities of the two plant species. Specifically, *Geobacter and Leptothrix* spp. dominated the Mayrhofen and Kilpisjärvi regions, while members of the *Clostridiales* preferred the Kilpisjärvi and Ny-Ålesund regions. Endosphere communities were enriched with *Goebacter* and *Clostridia*, whereas the soil communities were dominated with *Burkholderia* and *Leptothrix*.

Key words: *nifH,* arcto-alpine plants, *Geobacter*, *Clostridia*, *Leptothrix*

Introduction

Nitrogen (N) is considered to be the major limiting factor for both microbial and plant growth in arctic and alpine biomes. For example, Sistla et al. (2012) revealed - by N addition experiments - that microbial growth, enzyme synthesis and carbon allocation in tussock tundra soils during the arctic summer are N-limited. Furthermore, corroborating this finding, Wallenstein et al. (2009) observed overall decreases in microbial enzyme activities with the reduction of available soil nitrogen levels. In particular, the amount of available soil nitrogen is very low in low organic content mineral-rich tundra soils. In addition, a study of a chronosequence along the forefield of the Damma glacier in the Swiss Alps by Brankatschk et al. (2011) reported the presence of very low N nutrient contents in soils with deglaciation ages between 10 and 70 years. These soils also showed quite scarce vegetation. Furthermore, the authors observed no potential N-fixation activity and extremely low denitrification and nitrification rates. Finally, the *nifH* gene copy numbers were low, with the lowest numbers being found in the 10-year old soils. In contrast, a densely plant-covered 120-year old soil showed both higher enzyme activities and higher copy numbers of the *nifH*, *nirK* and *nirS* genes. Clearly, the older soils had regained the capacity to cycle nitrogen on the basis of a pioneer species based ecosystem build-up, in which plants found capacities to grow and, in turn, supported the evolved microbial communities and activity.

The N limitation is underlined by a higher demand for N by arctic plants compared to their temperate-climate relatives. Given the fact that arctic plants are adapted to low temperatures during the growing season and photosynthesis rates (at 68°N) are comparable to those at lower latitudes (Central Europe) (Chapin et al., 1987), higher amounts of enzymes (and so higher N) are required. For example, higher numbers of the key photosynthesis enzyme ribulose 1,5 bisphosphate (RuBiSco) are required to compensate for the slower kinetics at low temperatures. Thus, the tissues of arctic plants contain more N than those of plants in warmer climates (Weintraub and Schimel, 2005), underlining the demand for N availability. Clearly, in nascent ecosystems such as those forming in the low-nutrient arctic soils, provision of nitrogen is a key issue, as living systems critically depend on reduced nitrogen. Biological nitrogen fixation by free-living local bacteria (under which cyanobacteria), appears as a key strategy involved in the local primary production, yielding organic compounds containing reduced nitrogen. In the emerged systems, with plants in place as primary producers, the key nitrogen input may come from plant-symbiotic and/or associative nitrogen fixers, whereas nitrogen from the produced organic compounds is recycled/mineralized via deamination (yielding ammonia), followed by nitrification and denitrification reactions. Unfortunately, we currently lack

information as to the key organisms involved in these important steps of the nitrogen cycle in the Arctic.

In previous studies, we selected two plant species, *Oxyria digyna* and *Saxifraga oppositifolia,* as these are among the primary colonizers of the nutrient poor mineral soils that abound in both arctic and alpine regions. In these studies, we have found preliminary evidence for the contention that the soil bacterial communities in different arcto-alpine regions are region-specific, whereas the endophytic bacterial communities (using 16S rRNA gene as a proxy) are host plant specific (Kumar et al., 2016b). However, these host plant specific communities also include shared core taxa, which are associated with their host plants in all three climatic regions. Unfortunately, the role of these core taxa has been understudied, although, on the other hand, the data already provided glimpses of the presence of several potential nitrogen fixers in the core microbiome (Kumar et al., 2016b).

In this study, we place a focus on the nitrogen fixing bacteria in the two selected arcto-alpine plants, with the hypothesis that key potential nitrogen fixers identified as major members of the bacterial communities associated with these plants are indeed involved in local N fixation processes. In the light of the impossibility to measure local nitrogen fixation rates, we provide a thorough discussion of the likelihood of this tenet.

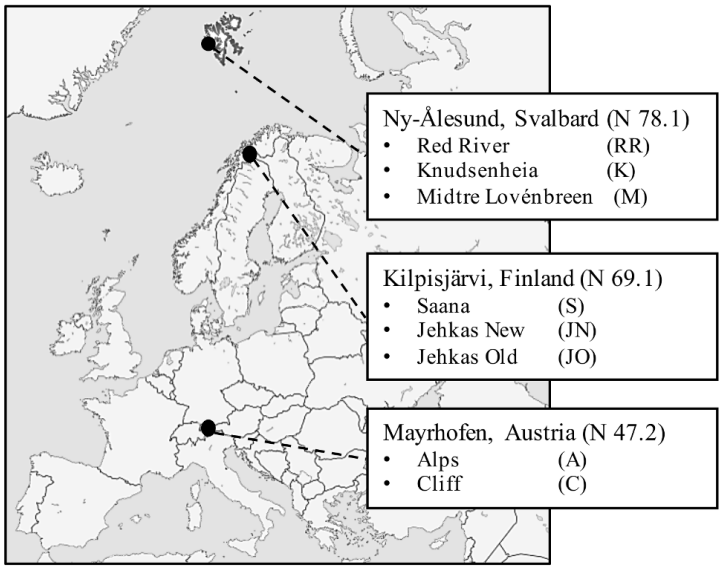

Figure 1 Sampling locations; Mayrhofen in Austrian Alps, Kilpisjärvi in low-arctic Finnish Lapland and Ny-Ålesund in high-arctic Svalbard archipelago

Materials and methods

Sampling and DNA isolation:

Samples were collected in June 2012 from three different regions, i.e. two in the arctic (Kilpisjärvi (KJ) and Ny-Ålesund (NÅ)) and one in the European Alps (Mayrhofen (MA)) (Figure 1). NÅ is located in the high Arctic, where soil temperatures do not rise above 10°C, whereas alpine MA sites and KJ low Arctic sites experience higher annual temperature fluctuations. Three replicates of bulk soil samples and three plant samples of each *O. digyna* and *S. oppositifolia* (with adhering rhizosphere soils) were collected from all sites. All samples were processed as specified by Kumar et al. (2016). In brief, after removing rhizosphere soil parts, plant roots were thoroughly washed with water and then surface sterilized by immersion into 3% sodium hypochlorite (3 min), followed by sterile double-distilled water (3 x 90 s). 80-100 mg of processed root sample were snap-frozen with liquid nitrogen and stored at -80°C for further DNA extraction and analysis. In addition, 250-300 mg of rhizosphere and bulk soil samples were stored at -80°C.

nifH library preparation and sequencing

The MoBio Power soil kit (MoBio, Carlsbad, CA, USA) and Invisorb Spin plant Midi kit (STRATEC, Biomedical AG, Germany) were used to extract DNA from soil and plant samples, respectively (Kumar et al., 2016b). The *nifH* gene was amplified using a nested approach. The first PCR was done with primers 19f/nifH3 (Yeager et al., 2004) and the second one with one forward primer nifH1f (as described by Zehr and Mcreynolds, 1989) and equimolar concentration of reverse primers nifH2r (Zehr and Mcreynolds, 1989) and nifH2 (Izquierdo and Nüsslein, 2006). The first PCR reaction had 2 µl of template DNA, 1x PCR buffer, 2.5mM of MgCl2, 1 mg/ml of BSA, 0.4 mM dNTP's, 0.9 µM of each primer and 2500 U/ml of GoTaq DNA ploymerase (Promega, WI, USA) in a 30 µl reaction volume. For the second PCR, 1 µl of amplified product from the first PCR was added along with 1x PCR buffer, 1 mM of MgCl2, 1 mg/ml of BSA, 0.2 mM dNTP's, 0.9 µM of each primer and 1250 U/ml of GoTaq DNA ploymerase (Promega) in a 30 µl reaction volume. Amplifications for both PCR reactions were performed as follows: 2 min denaturation at 95°C followed by 35 cycles of denaturing, annealing and extension at 95°C for 1 min, 51°C (first PCR)/ 53°C (second PCR) for 1 min and 72°C for 1 min, respectively. Final extension was carried out at 72°C for 2 min.

Sequence libraries were then prepared by running a third PCR to attach the M-13 barcode using the system developed by Mäki et al. (2016). An aliquot (1 µl) of the second PCR was re-amplified using the barcode-attached M13

system as forward primer and nifH2r/nifH2-P1 with adaptor A as reverse primer. PCR mix and conditions were similar to those described for the second PCR, except that only 8 cycles were used for amplification. The products were then purified using the Agencourt AMPure XP PCR purification system (Beckman Coulter, CA, USA), followed by quantification with a Qubit Fluorometer (Invitrogen). Equivalent DNA quantities of each sample were then pooled, size-fractionated (size selection range of 350-550 bp) using Pippin Prep (Sage Science, MA, USA) 2% agarose gel cassette (Marker E) according to the manufacturer's protocol. The size-fractioned libraries were sequenced using Ion 314 chip kit V2 BC on an Ion Torrent PGM machine (Life Technologies, MA, USA) in the Biocenter, Oulu, Finland.

Bioinformatics and statistical analysis

All reads from the Ion torrent sequencing were processed using QIIME (Caporaso et al., 2010) and Fungene pipelines (Fish et al., 2013), as described by Zhang et al. (2015). All sequences with quality scores below 22 and lengths below 240bps were removed. USEARCH (Edgar, 2010) algorithm in de novo was used for chimer removal. After chimera removal, all nucleotide sequences were translated into protein sequences and frameshift-corrected with Framebot (length cutoff = 80 AAs), aligned using HMMER3 Aligner (with *nifH* as representative gene) and then clustered by RDP mcClust (90% similarity) in Fungene. The resulting cluster file was then converted into an OTU table by RDP cluster file formatter in R (version 3.2.5; https://www.r-project.org/). Then, OTUs were then assigned to taxa by FunGene FrameBot, annotations. The OTUs were grouped into OPUs by clustering with the same reference sequence. Taxonomic affiliations of 12 OPUs were complemented by P-BLAST at NCBI.

The OTU table was normalized and OPU abundances were square-root transformed prior to community analysis with the PRIMER 6.1 (http://www.primer-e.com/) software package. In addition, PRIMER 6 was also used to determine the similarities between the samples (ANOSIM) and to assess the dissimilarity between dominating OPUs from different regions or compartments (SIMPER). PCoA graphs were also plotted with PRIMER 6 based on Bray-Curtis dissimilarity matrix. Venny (http://bioinfogp.cnb.csic.es/tools/venny/) was used for constructing a Venn diagram showing similarities and dissimilarities across the geographic region.

Results

We successfully produced pure total microbiome DNA from 165 samples taken from bulk soil, rhizosphere soil and the endosphere of *O. digyna* and *S.*

oppositifolia from the Mayrhofen, Kilpisjärvi and Ny-Ålesund regions. Then, PCR amplifications were performed with the 19f/nifH3r and nifH1f/nifH2r and nifH2 primers (amplifying 350 bp of the *nifH* gene). This nested approach was optimized by us to have maximum coverage of all phylogenetic diazotrophic groups based on the review by Gaby and Buckley (2012). All quality-trimmed *nifH* sequences were translated to the respective amino acid sequences. These were used for clustering, yielding 296 OTUs (at 90% amino acid sequence identity). These 296 OTUs were further clustered based on alignments with *nifH* protein reference sequences in the RDP FunGene *nifH* database. This clustering finally resulted in 129 units, which were denoted 'operational phylogenetic units' (OPUs).

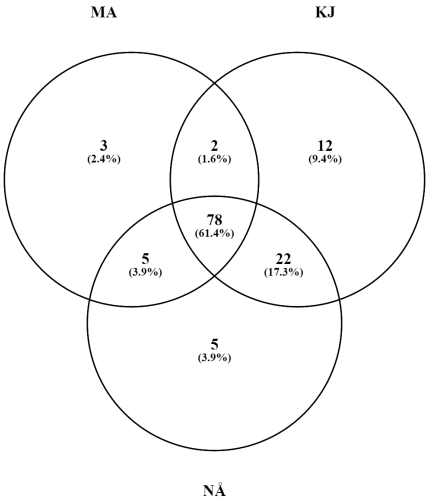

Figure 2 Nitrogen fixing OPUs (*nifH* gene sequences clustered with the closest reference sequences) in the three geographic regions. Figure legends: MA - Mayrhofen, the Alps, KJ - Kilpisjärvi, low Arctic, NÅ - Ny-Ålesund, high Arctic

Distribution of nifH OPUs over geographic region

Seventy-eight of the 129 OPUs (62%) were present across all three geographic regions used in this study. Thirty-nine OPUs (31%) were present in either one or both arctic regions, i.e. NÅ and KJ, whereas these were missing from MA in the alpine region (Figure 2). Ten OPUs (7%) were unique to MA or shared between MA and either one of the two arctic regions (Figure 2)

Diazotrophic communities – an overview

Overall, we detected high diversities of potential nitrogen fixing bacteria (PNFB) in all our samples. Remarkably, we detected *nifH* genes from the

bacterial taxa *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Chlorobi*, *Bacteroidetes*, *Cyanobacteria* and *Verrucomicrobia*. The most abundant *nifH* gene sequences in the dataset clustered with *nifH* gene sequences from the *δ-Proteobacteria* (genera *Geobacter* and *Desulfotignum*), *Clostridiales* (*Clostridium, Desulfosporosinus* and *Acetobacterium*), *β-Proteobacteria* (*Burkholderia* [*Burkholderiales*] and *Leptothrix* [*Comamonadaceae*]), *γ-Proteobacteria* (*Bradyrhizobium* [*Rhizobia*]).

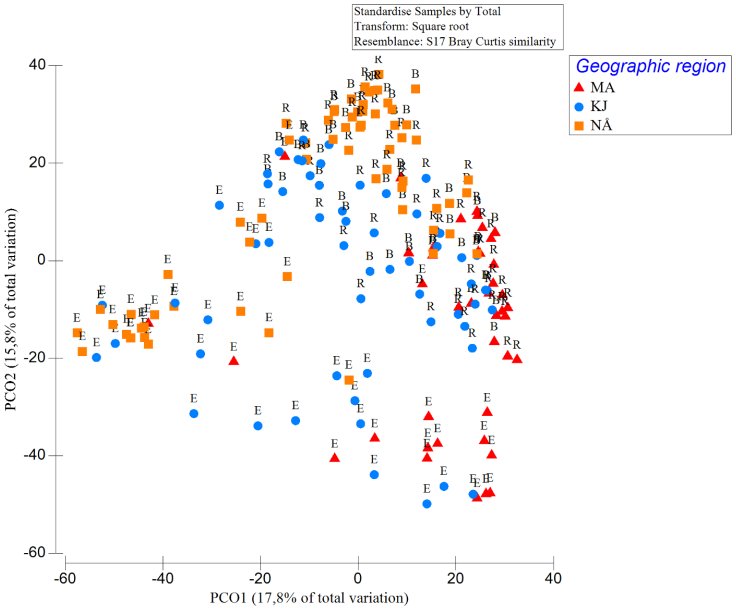

Figure 3 Principal Coordinate Analysis (PCoA) plots based on Bray-Curtis similarity matrix of PNFB communities (*nifH*-based OPU sequences as proxy species) from bulk soil (B), rhizosphere soil (R) and endosphere (E) from three climatic regions Mayrhofen (MA), Kilpisjärvi (KJ) and Ny-Ålesund (NÅ)

Community structures of PNFB

Community structures of the PNFB were analyzed at the OPU level across all samples. We observed no influence of plant species on the community structures of either rhizosphere or endosphere PNFB (Supplemental figures S1, S2). ANOSIM further confirmed that Geographic region (rhizosphere - R = 0.57; endosphere – R = 0.36) was the primary determinant of the plant-associated PNFB, followed by plant species (rhizosphere - R = 0.15; endosphere – R = 0.20). Considering the negligible effect of plant species, we combined the *O. digyna* and *S. oppositifolia* communities and analysed the data set based on 'Compartment' (bulk soil, rhizosphere soil, endosphere) across the three geographic regions. ANOSIM (Table 1) and PCoA ordination showed (Figure 3) that both geographic region and compartment strongly impacted the PNFB community structures. The endosphere PNFB

communities were significantly different from those from both soil compartments, while we detected only small differences between bulk and rhizosphere soil PNFB communities. Curiously, the endosphere communities were less similar to the rhizosphere than to the bulk soil communities (Table 1). The NÅ communities (analyzed across the different compartments) were relatively more different from both the KJ and MA communities than the latter two from each other (Table 1).

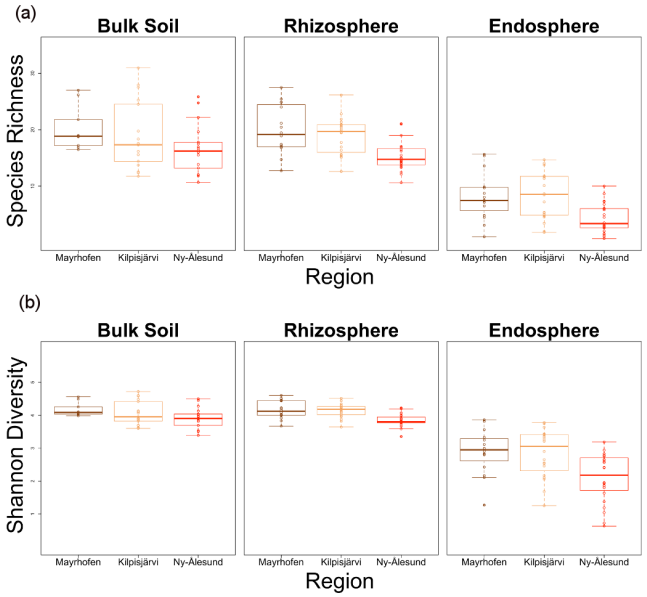

Figure 4 Species richness (a) and shannon diversity (b) of PNFB communities in different regions and compartments. Diversity indices were calculated using *nifH* gene OPUs as a proxy for species. Figure legends: MA - Mayrhofen; KJ - Kilpisjärvi; NÅ - Ny-Ålesund; B - bulk soil; R - rhizosphere soil, E – endosphere

Species richness as well as diversity of PNFB communities is lowest in the high Arctic

The species richness (SR) and Shannon diversity (SD) values of the PNFB communities (based on the *nifH* gene amplicon OPUs as a proxy) were highest in the bulk soils, followed by the rhizosphere soils and the endosphere samples. The diversities in the endosphere samples were significantly lower than those in the two soil compartments (Figure 4a, 4b).

The differences in the diversity and richness measures between the three geographic regions were estimated separately for each compartment. Species richness and diversity values of bulk soil communities were both highest in the MA, followed by the KJ and NÅ regions. However, in the plant-associated compartments (rhizo- and endospheres), both species richness and

diversity values were highest in the KJ communities, followed by the MA and NÅ communities (Figure 4a, 4b).

Table 1 One-way ANOSIM statistics for comparisons of PNFB communities according to geographic region (MA = Mayrhofen, KJ = Kilpisjärvi and NÅ = Ny-Ålesund), sample compartment (E = endosphere, R = rhizosphere soil and B = bulk soil) or plant species (O = *O. digyna*, S = *S. oppositifolia*). using OPU similarity values calculated based on Bray-Curtis resemblance matrix with R-value and significance value in % (in brackets). Significance values are 0.1% unless it is mentioned in the table

Region				
	All Samples	E	R	B
All Samples		0.302	0.505	0.406
MA vs KJ	0.139	0.238	0.346	0.284
MA vs NÅ	0.249	0.48	0.626	0.291
KJ vs NÅ	0.179	0.186	0.535	0.530
Compartment				
	All regions	MA	KJ	NÅ
B vs R	0.049	0.194 (2%)	0.127 (1.6%)	0.092 (3.1%)
B vs E	0.258	0.26 (0.7%)	0.578	0.389
R vs E	0.359	0.436	0.459	0.54
Plant species (O vs S)				
Endosphere	All regions	MA	KJ	NÅ
	0.105 (0.2%)	0.18	0.230	0.132 (2.1%)

Different bacteria dominate soil- and plant-associated PNFB communities in different regions and compartments

SIMPER analysis confirmed the findings from our initial taxonomic analyses. Thus, different bacterial taxa dominated the PNFB communities in different geographic regions. The ***bulk and rhizosphere soil*** PNFB diverged from the endosphere ones. *Leptothrix* and *Burkholderia* type *nifH* sequences dominated both the bulk and rhizosphere soil communities across all three regions (Figure 5a). Additionally, OPUs clustering with *nifH* sequences from the *Opitutaceae* (*Verrucomicrobia*) were highly abundant in the KJ bulk soils, whereas *Acetobacterium bakii* (*Clostridiales*) *nifH* sequences were predominantly present only in the rhizosphere soils from NÅ. Compared to MA and KJ, *Geobacter*-type *nifH* sequences were detected only at very low relative abundances in NÅ, while the soil communities were dominated by the genera *Burkholderia, Leptothrix* and species *Nostoc punctiforme* and *Nodularia spumigena* (*Cyanobacteria*).

The ***endosphere*** communities were often enriched with anaerobic nitrogen fixers from the genera *Geobacter* and *Clostridium* (Figure 5a). Thus the MA communities were strongly dominated by *nifH*-based OPUs related to several *Geobacter* species, including *G. lovelyi, G. bemidjiensis* and *G. uraniireducens* (Figure 5b). Specifically, six out of seven key OPUs (SIMPER analysis) of these communities represented the genus *Geobacter* (Table 2). In contrast, the main OPUs structuring the NÅ endophytic PNFB communities represented

the genera *Clostridium* (three OPUs, including *C. akagii* and *C. pasteurianum)*, *Leptothrix* (*Comamonadaceae*, two OPUs) and unidentified_bacterium (OPU 1364). While *Clostridium*-like OPUs were virtually absent in the MA endosphere communities and *Geobacter*-type OPUs likewise in NÅ, the major *nifH* OPUs in the KJ endosphere communities represented both the genera *Clostridium* and *Geobacter*, as well as *Bradyrhizobium* and *Burkholderia* (Table 2; Figure 5b). Specifically, OPU 3027 (*Bradyrhizobium sp.*) was predominantly found in the KJ endosphere samples. The endosphere and soil communities in NÅ were more dissimilar from each other (ANOSIM R=0.74, 0.1%) than the ones in KJ or in MA (ANOSIM R=0.68, and R=0.63 respectively).

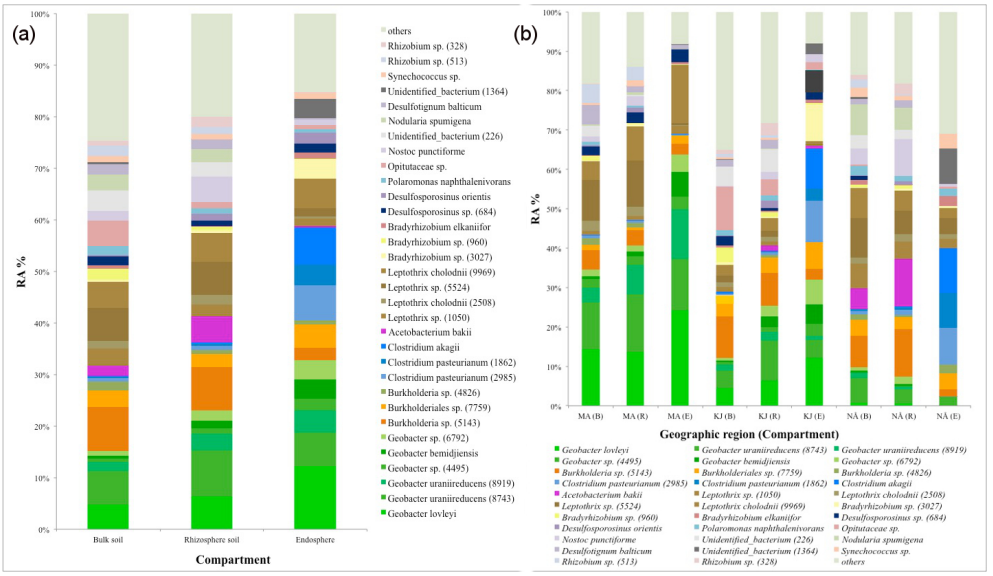

Figure 5 Average relative abundance of PNFB communities plotted based on sample compartment (a) and geographic region separated based on compartment (b). Only selected major PNFB OPUs were shown, and OPUs with similar species/genus were makred with OPU numbers (in bracket)

Geobacter and Bradyrhizobium diazotrophs are differentially enriched in S. oppositifolia and O. digyna endospheres

As stated above, the compartment, in particular the endosphere-soil division, was the major determinant of the PNFB communities, with no strong host-plant specificity. However, we detected some trends in the affiliation of several OPUs with particular plant species. OPUs representing *Bradyrhizobium*, *Burkholderia* and OPU 1_783 (*nifH* from an unidentified bacterium) were consistently present in high abundances in the *O. digyna* endosphere samples (Figure 6), whereas *S. oppositifolia* endosphere communities were, instead, more enriched with *Geobacter* related OPUs, especially in the MA and KJ regions.

Furthermore, the *S. oppositifolia* endosphere communities harbored relatively more OPUs related to *Desulfosopronius* (*Clostridiales*) in KJ and *Clostridium* and *Desulfuromonas* related OPUs in NÅ, all in comparison to *O. digyna* (Figure 6).

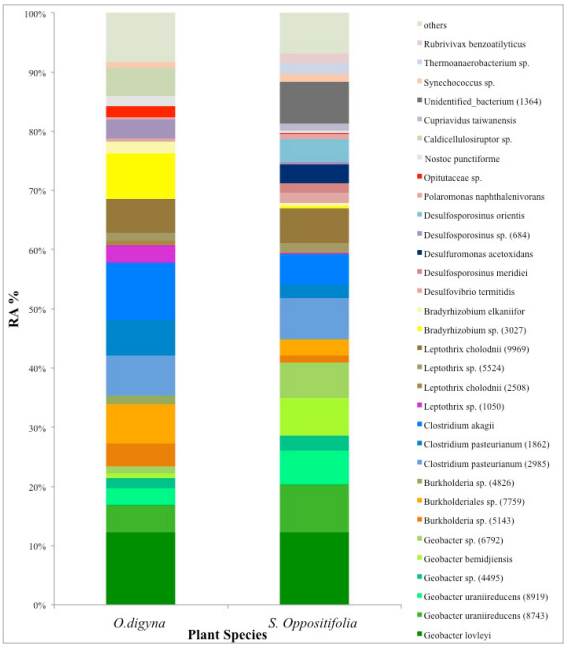

Figure 6 Average relative abundance of PNFB communities plotted based on plant species O. digyna and S. oppositifolia across all three regions. Only selected major PNFB OPUs were shown, and OPUs with similar species/genus were marked with OPU numbers (in bracket)

Discussion

Using the *nifH* gene as a proxy, we here analyzed the diversity and community composition of PNFB in two pioneer plant species growing in low-nutrient soils in three arcto-alpine regions. Overall, we detected high diversities across the samples, with *nifH* genes from seven different bacterial phyla being present. With respect to the breadth of detection, we argue that the primers selected theoretically allow to obtain maximum coverage from all the *nifH* phylogenetic groupings (Gaby and Buckley, 2012). Indeed, our libraries had good phylogenetic coverage, with all OPUs representing seven phyla, including representatives from *nifH* clusters I, II and III. In specific, the PNFB communities were largely dominated by *nifH* gene types from the *α-, β-, γ-* and *δ-proteobacteria* and the *Clostridiales*. The *δ-proteobacterial* genus *Geobacter*, the *β*-proteobacterial genera *Burkholderia* and *Leptothrix,* as well as *Clostridia (*from the *Firmicutes)* were the most abundant. With the exception of *Burkholderia* and *Leptothrix*, the dominant diazotrophs in these communities have been mostly described from anoxic soils and sediments (Dang et al., 2013; Rösch et al.,

2002; Wu et al., 2009; Yeager et al., 2004; You et al., 2005). This points at a key suggestive finding of this study, i.e. the likely prevalence of conditions enabling nitrogen fixation under anoxia in most of the soils. Both *O. digyna* and *S. oppositifolia* are known to be able to tolerate waterlogging in soil. The two are common snow patch species, as known from observations in the low-arctic sites in KJ. We sampled all sites relatively early in the growth season, when the plants were flowering, which was relatively shortly after snowmelt at these sites. Only the NÅ region had permafrost, with KJ having seasonal and patchy permafrost, with snow often melting away as late as mid-July. The MA site soils were, however, relatively less water-logged than the soils from the arctic sites.

Table 2 Data based on SIMPER analysis of normalized and square root transformed major OPU/species driving the dissimilarities between different regions in the endosphere samples. All OPUs which contribute to 90% dissimilarity were selected and presented in the table

OPU/Species	Average Abundance	Average Similarity	Contribution to Dissimilarity %
Average dissimilarity = 30.23 (Mayrhofen)			
*Geobacter lovleyi*_SZ	3.92	9.14	30.25
Geobacter uraniireducens_Rf4	2.96	6.45	21.35
Geobacter uraniireducens sp	2.62	5.27	17.43
Geobacter CP001089	1.38	2.86	9.47
*Geobacter bemidjiensis*_Bem	1.57	1.61	5.32
*Geobacter*_2397398_2398267_CP001124	1.37	1.54	5.08
Bradyrhizobium elkanii	1.22	0.84	2.79
Average dissimilarity = 22.96 (Kilpisjärvi)			
*Clostridium pasteurianum*_4_1-like	2.23	3.26	14.19
*Geobacter*_2397398_2398267	2	2.9	12.62
Bradyrhizobium	2.27	2.76	12.03
Clostridium akagii	1.73	2.33	10.13
Geobacter_lovleyi_SZ	1.95	2.31	10.05
Geobacter_bemidjiensis_Bem	1.4	1.77	7.73
Clostridium_pasteurianum_1_	1.14	1.69	7.37
*Geobacter*_CP001089	0.97	1.11	4.82
*Geobacter uraniireducens*_Rf4	0.98	0.78	3.38
Bradyrhizobium elkaniifor	0.58	0.63	2.74
*Desulfosporosinus orientis*_DSM_765	0.86	0.47	2.05
*Burkholderiales*_321041_321922	0.95	0.39	1.69
*Burkholderia*_sp_Ch1-1_ctg00023	0.68	0.38	1.65
Average dissimilarity = 18.71 (Ny-Ålesund)			
*Clostridium pasteurianum*_4_1-like	2.48	4.56	25.1
Clostridium akagii	2.31	3.47	19.09
Bradyrhizobium elkaniifor	2.07	3.25	17.88
1_783_AB094963	1.2	1.73	9.55
*Opitutaceae*_bacterium_TAV2_ctg796	0.88	1.31	7.23
Clostridium pasteurianum	1.21	0.88	4.87
*Nostoc punctiforme*_PCC_73102	0.44	0.5	2.77
*Polaromonas naphthalenivorans*_CJ2	0.49	0.25	1.39
*Leptothrix*_161893_162774_CP002016	0.59	0.24	1.33
*Leptothrix cholodnii*_SP-6	0.44	0.24	1.3

Compared to reports from high-organic-matter tundra soils from Arctic or alpine sites, where aerobic proteobacterial diazotrophs reportedly dominate (Tai et al., 2013), our soils were mostly enriched in *δ-proteobacteria* and *Clostridiales* (depending on region and compartment). The current data, where we found no influence of plant species (*O. digyna* and *S. oppositifolia)* on the PNFB communities in the endosphere were different from those on the 16S bacterial community structures (Kumar et al., 2016b). However, a previous study on fungal community structures was in line with the contention that plant species was a minor driver (Kumar et al., 2016a). In addition, both fungal and PNFB community structure were influenced by geographic region. We here hypothesize that the two plant species most likely 'behave' in a similar manner in the soils, for instance secreting similar compounds that attract the diazotrophs as well as fungi. Possibly, although unproven, the diazotrophs are tightly associated with the selected fungi, or each of the three components of the system are similarly selected to make part of it.

As mentioned, clear regionality was also evident in the distribution of the diazotrophic communities. Thus *Geobacter* was abundantly present in the sites from MA and KJ, at altitudes of about 2400m and 900m above sea level. This was in line with the observation of this taxon in high mountain ranges where it could metabolize under both oxic and anoxic conditions (Ciccazzo et al., 2016). *Geobacter* has also been detected in *nifH* defined communities from switchgrass roots in oxic temperate-climate prairie ecosystems (Bahulikar et al., 2014), indicating adjustment to temperate and environmental plasticity to utilize both aerobic and anaerobic soil conditions.

Moreover, *Clostridiales,* an anaerobic taxon (*Clostridium* and *Acetobacterium*), were virtually absent from the MA samples, whereas they increased in abundance towards the north. In addition, this may reflect the different bioclimatic conditions between MA and NÅ, as discussed.

It should be noted, though, that - even in NÅ - *Clostridium* was mainly restricted to the endosphere, which is actually in agreement with our analysis of total bacterial communities of the same plants (based on the16S ribosomal RNA [rRNA] gene) (Kumar et al., 2016b). Specifically, we observed higher relative abundances of *Clostridium* with increasing latitude and restricted to the endospheres of both plant species. Such *Clostridium* PNFB (predominantly observed in the endosphere) also showed endosphere specificity in the previous 16S rRNA gene based analysis (Kumar et al., 2016b). In addition, Given et al. (personal communication) observed 16S rRNA sequences of *Clostridium* spp. in seeds of *O. digyna,* indicating possible vertical transmission. Both *Geobacter* spp. and *Clostridium* spp. have been detected as diazotrophs in rice roots in flooded rice paddy fields (Hardoim et al., 2012; Sessitsch et al., 2012).

The diversity of PNFB communities was lowest in the high-arctic NÅ sites, with a similar trend being detectable in all (i.e. the bulk soil and plant associated (rhizo- and endosphere)) compartments. While the bulk soil communities were most diverse in the alpine sites in MA, the plant associated communities were most diverse in KJ, which is in contrast to the bacterial and fungal communities from the same sites, where we observed the highest diversity in rhizosphere samples (Kumar et al., 2016a; 2016b).

Corroborating the previous 16S rRNA based analyses (Kumar et al., 2016b), *O. digyna* and *S. oppositifolia* shared some plant specific PNFB OPUs. OTUs previously identified as 'core' or as part of the endosphere community, e.g. *Bradyrhizobium*, *Burkholderiales*, *Comamonadaceae* and *Clostridia* (Kumar et al., 2016b) were also found to make part of the PNFB endosphere communities. Thus, such N fixers are often key dominant factors in the plant-associated microbiomes, indicating the importance of (plant-associated) nitrogen fixation for these plants. It may well be that plants recruit these bacteria from the soil, allowing N fixation to occur in the 'protected' environment offered by the plant. The outcome of this selective process might be improved plant growth in the highly N limited soils. Furthermore, this signifies importance of N fixating bacteria for plant fitness and growth in these conditions.

Concluding, the nature of the PNFB communities found at two plant species in three different cold-climate geographic regions points to selective processes that operate at different scales, i.e. that of compartment as well as of region. Plant type appeared to have a lesser influence, with the exception of some OPUs from the order *Burkholderiales* (*Burkholderia sp.*) in *O. digyna* and the order *Clostridiales* (*Desulfosporsinus sp.*) in *S. oppositifolia*. These organisms were also found to be enriched in the (16S rRNA defined) endosphere communities from the respective plants. Finally, on the basis of the predominance of anaerobic nitrogen fixers, we surmised that local conditions allowing nitrogen fixation might have been prevalently anoxic in the two northern regions, whereas those in the alpine region may have been more variable (oxic or anoxic).

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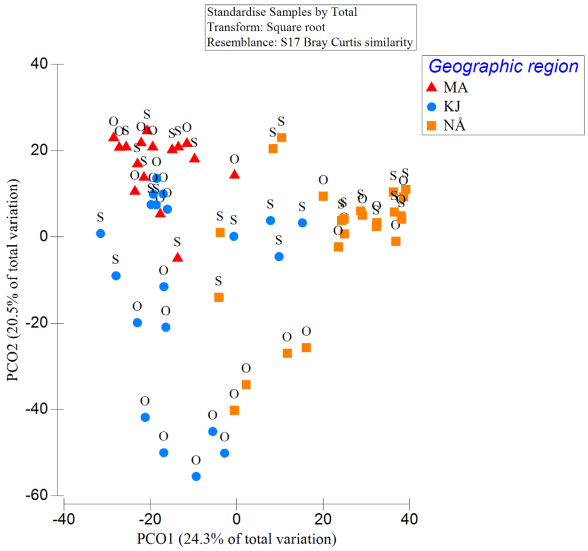
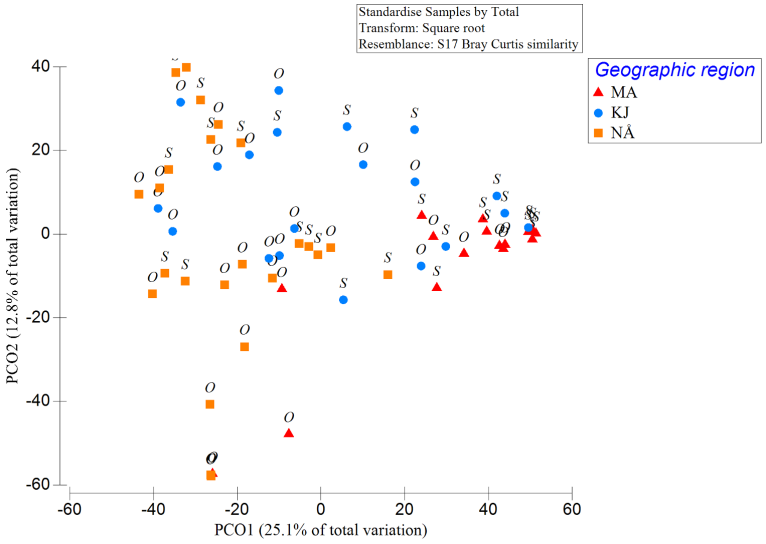


Supplemental figure S1 Principal Coordinate Analysis (PCoA) plots rhizosphere soil PNFB communities (*nifH*-based OPU sequences as proxy species) from O. digyna (O) and S. oppositifolia (S) plant species from three climatic regions Mayrhofen (MA), Kilpisjärvi (KJ) and Ny-Ålesund (NÅ)



Supplemental figure S2 Principal Coordinate Analysis (PCoA) plots endophytic PNFB communities (*nifH*-based OPU sequences as proxy species) from O. digyna (O) and S. oppositifolia (S) plant species from three climatic regions Mayrhofen (MA), Kilpisjärvi (KJ) and Ny-Ålesund (NÅ)